regions within the exchanger's cytoplasmic domain, of which the XIP region shown to be involved in the rate and extent of Na^+-dependent inactivation (I_1). Although the XIP region is highly conserved amongst NCX isoforms, distinct I_1 phenotypes exist. To better define the role of this region, we constructed chimaeric NCX1:NCX2.1 proteins with their respective XIP regions interchanged as well as amino acid substitutions within the XIP region to examine the more subtle aspects of phenotypic differences between NCX1.3 and NCX2.1. Mutant exchangers were expressed in *Xenopus* oocytes, and outward Na^+-Ca^{2+} exchange activity was assessed using the giant, excised patch clamp technique. Substitution of the XIP region of NCX1.4 with the corresponding region from NCX2.1 caused an apparent loss of I_1 whereas a reduction in the extent of inactivation and a 15-fold increase in the rate of recovery from I_1 were observed in the NCX1.3 - XIP2 chimaera. Similarly, substitution of charged amino acids within the XIP region in NCX1.3 caused a slight increase in the rate of recovery, equivalent to that observed for NCX2.1. Thus, non-conserved residues in the XIP region may be essential in maintaining the structural stability of the Na^+-dependent inactive state of NCX1. Furthermore, the XIP region must interact with other regulatory domains of the protein, such as the mutually exclusive exon, thereby contributing to the structure-function relationship as well as the regulatory phenotype of each Na^+-Ca^{2+} exchanger variant and isoform.

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The Role of Phospholamban Cysteines in the Activation of the Cardiac Sarcoplasmic Reticulum Calcium Pump by Nitroxyl

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Phospholamban (PLN) is an integral membrane protein that regulates the Ca^{2+} pump (SERCA2a) in cardiac sarcoplasmic reticulum (CSR). Phosphorylation of PLN in response to β-adrenergic stimulation enhances cardiac inotropy by increasing CSR Ca^{2+} uptake. Nitroxyl (HNO), a new candidate drug therapy for congestive heart failure, improves overall cardiovascular function by increasing Ca^{2+} release and re-uptake in CSR through a direct interaction with RyR2 and SERCA2a, respectively. Using insect cell ER microsomes expressing SERCA2a +/− PLN (WT and Cys → Ala mutant) we have shown that activation of SERCA2a by HNO is PLN-dependent and entails covalent modification of PLN cysteines. Although HNO stimulates SERCA2a activity by uncoupling PLN from SERCA2a, the role of the cysteine residues in the activation mechanism is not completely understood. We propose that HNO, a thiol oxidant, modifies one or more of the three PLN cysteine residues (C36, C41, C46), affecting the regulatory potency of PLN toward SERCA2a. Examples include intra-molecular disulfide cross-links within single PLN molecules or inter-molecular disulfide cross-links between PLN molecules or PLN and SERCA2a. To test this hypothesis, we have constructed a series of PLN mutants containing single, double and triple cysteine substitutions (alanine replacing cysteine). Each of these mutant PLNs will be co-expressed with SERCA2a in insect cells and cell microsomes will be treated with Angeli's salt (an HNO donor) to determine which cysteine residue(s) are essential for activation monitored by enzyme assay and fluorescence spectroscopy of SERCA2a. The results show that intermolecular PLN disulfides play a minor role in activation by HNO. Studies with the Cys → Ala mutations will be useful in determining which cysteine pairs in PLN contribute to intramolecular disulfide cross-links leading to the relief of PLN inhibition and SERCA2a activation.

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Site Directed Mutagenesis of Human GLTP: Role of Tryptophan Residues

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Glycolipid transfer proteins (GLTPs) are small, soluble, ubiquitously expressed proteins that selectively accelerate the intermembrane transfer of glycolipids in vitro. Mammalian GLTPs (209 aa) are intrinsically fluorescent by virtue of having three tryptophans and ten tyrosines. The crystal structure of human GLTP (glycolipid-bound form) reveals the importance of W96 in the glycolipid liganding site where its aromatic indole ring acts as a stacking platform that facilitates hydrogen bonding of the initial ceramide-linked sugar with Asp48, Asn52, and Lys55. To gain insights into W96 functionality and to define the role of the other two Trp residues (i.e., W85 & W142), three GLTP Trp mutants (W96Y, W85Y-W96F, W96F-W142Y) were constructed by QuikChange™ site-directed mutagenesis, overexpressed (pET-30) in E. coli, purified by metal ion affinity and FPLC size exclusion chromatography, and characterized by glycolipid transfer activity measurements and by fluorescence and CD spectroscopy. Compared to wtGLTP, the single Trp mutant, W96Y, retained 65% activity; whereas the double Trp mutants, W85Y-W96F & W96F-W142Y, retained 22% and 110% activities. Quenching with acrylamide and potassium iodide at physiological ionic strength resulted in linear Stern-Volmer plots, suggesting accessibility of emitting Trp residues to soluble quenchers and consistent with wtGLTP native folding. However, CD measurements revealed significant differences in the secondary structure of W85Y-W96F-GLTP compared to wtGLTP; whereas W96F-W142Y-GLTP and W96Y-GLTP retained native secondary structure. We conclude that the negative consequences of conservative mutation of Trp 85 suggest a crucial role in proper folding of GLTP; whereas, the tolerance of Trp96 and Trp142 for conservative, but not radical, mutation is consistent with specialized roles in GLTP function, i.e. glycolipid liganding and membrane interaction. [Support: NIH/NIGMS GM45928 & GM34847, NIH/NCI CA121493, The Hormel & Mayo Foundations].

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Effects Of Gap Junction Blockers On The $P2x_7$ Receptor

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Peritoneal macrophages express the $P2X_7$ receptor, which opens a pore in the membrane after long exposure with ATP, allowing passage of molecules up to 900 Daltons. It has been argued that activation of $P2X_7$ receptor leads to opening of an independent pore entity, not structurally related to the $P2X_7$ receptor. Based on results with connexin knock outs and pharmacological manipulation with known gap junction blockers, some groups have included connexins and pannexins, the gap junction-forming proteins in vertebrates, as reliable candidates to provide for the large permeation pores associated with $P2X_7$ activation. In the present study we performed electrophysiological (whole cell patch clamping recordings) and permeabilization assays (optical analysis and FACS analysis) in which both efficacy and specificity of some gap junction blockers were tested at conditions of putative $P2X_7R$ activation by ATP. ATP generated a current in a nA levels that was blocked by well know P2X blockers as BBG, KN-62 and oxidized ATP, in contrast the junction blockers did not interfere with these effect. More than that, the up take assays showed similar results to the patch clamp experiments, none of the junction blockers was able to block the up take of ethidium bromide or propidium iodide. Our results indicate that well-known gap junction pharmacological blockers do not interfere with current generation or dye uptake after activation of $P2X_7$ receptor. Taken together, our data strongly suggest that the high permeability pore evident at prolonged $P2X_7$ activation does not correspond to connexin or pannexin hemichannels in peritoneal macrophages.

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Qm Simulation Of Binding Site In P-type Atpases

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P-type ATPases are enzymes that stablish cation gradients across biologcal membranes where ions are transported using the energy from hydrolysis of ATP. The Na,K-ATPase and SERCA1a transport 3 Na+/ 2 K+ and Ca2+ , respectively, against a concentration gradient for each ATP molecule hydrolyzed. The two enzymes have very high sequence homology and show structural similarities in the binding site of the ions. This raises questions concerning the selectivity of the different structural basis and how each protein select its specificion. The present study aims at characterising this selectivety.
In order to understand the specificity of the enzymes, we investigate the coordination site in the two crystal structure of SERCA1a(PDB ID: 1SU4) and Na+/K+ ATPase(PDB ID: 3B8) by constructing models of the active site. The models are constructed using analogs of the amino acids from the first and second solvation shell. We optimise the geometry of the models by constraining the C-alpha atoms using semi-empirical methods such as PM6 and AM1. The energies and geometries are investigated further using ab initio, restricted Hartree Fock, and density functional theory with the B3LYP functional using the basis set 6-31G(d,p)*. We substitute Ca2 ± ions into the binding pocket of the Na+/K+ ATPase model to investigate how the specificity is related to the coordination environment. Furthermore, the protonation state of side chains coordinating the ions are investigated in order to estimate the influence of pH changes on the binding of ions in the two pumps.

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Substrate Specificity And Peptide Bond Selectivity In Rhomboid Intramembrane Proteases

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